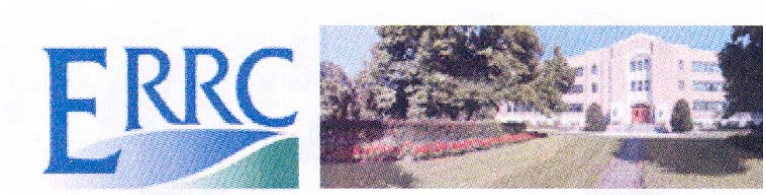

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Title: Effect of Temperature on the Radiation Resistance of Virulent Yersinia enterocolitica

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Effect of temperature on the radiation resistance of virulent *Yersinia enterocolitica*☆

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Abstract

Yersinia enterocolitica, a food-borne pathogen, can be eliminated from meat using ionizing radiation. Commercial facilities may irradiate meat at refrigeration or frozen temperature, or packed in dry ice if the facility does not have refrigeration capabilities. The effect of temperature on the radiation resistance of *Y. enterocolitica* that contained the 70 kb large virulence plasmid was determined. A mixture of four *Y. enterocolitica* strains was inoculated into ground pork, which was then vacuum-packed, equilibrated to refrigeration or sub-freezing temperatures, and irradiated to doses of 0.2, 0.4, 0.6, 0.8, and 1.0 kGy. The D_{10} value, the radiation dose required to reduce the number of viable *Y. enterocolitica* by 90%, increased as product temperature decreased with values of 0.19, 0.19, 0.21, 0.40, 0.40, 0.38, and 0.55 kGy being obtained at +5, 0, −5, −10, −15, −20 and −76 °C, respectively. Meat product temperature should be considered when selecting a radiation dose required for elimination of *Y. enterocolitica*.

Keywords: *Yersinia enterocolitica*; Ionizing radiation; Temperature; Pork

1. Introduction

Yersinia enterocolitica causes an estimated 96,000 cases of food-related illness in the United States annually (Mead et al., 1999). It is considered to be a pathogen of concern by the pork processing industry in the United States and is easily isolated from retail pork products (Davies, 1997). Like *Listeria monocytogenes*, *Y. enterocolitica* is capable of growth at refrigeration temperatures (Sutherland & Bayliss, 1994). The virulence of individual *Y. enterocolitica* strains is linked to the presence of a 70 kilobase virulence plasmid that encodes genes for a type III secretion channel and host immune suppression factors (Cornelis et al., 1998). Because of these characteristics, *Y. enterocolitica* is of particular interest as a food-borne pathogen.

Red meats, including pork, can be pasteurized using ionizing radiation doses up to 4.5 kGy for refrigerated product or 7.0 kGy for frozen product (Federal Register, 1997). Ionizing radiation can eliminate *Y. enterocolitica* from refrigerated pork products (Grant & Patterson, 1991; Kamat, Khare, Doctor, & Nair, 1997; Shenoy, Murano, & Olson, 1998; Sommers & Bhaduri, in press). Studies have shown that the radiation resistance of food-borne pathogens increases with decreasing temperature (Lopez-Gonzalez, Murano, Brennan, & Murano, 1999; Thayer & Boyd, 1992, 1995, in press). The radiation resistance of virulence plasmid containing *Y. enterocolitica* at subfreezing temperatures has not adequately been determined.

Organoleptic changes in meat irradiated at subfreezing temperatures are smaller than those irradiated at refrigerated temperatures at the same dose, making the practice of irradiation at subfreezing temperature an attractive process. The selection of temperature during irradiation would therefore be dependent on the individual product characteristics. However, the microcidal effect of ionizing radiation at subfreezing temperature is weaker than at refrigerated temperatures. Therefore, the radiation dose used to eliminate a specific pathogen should be higher in frozen meat than in non-frozen meat.

Because many commercial irradiation facilities lack the ability to refrigerate product or maintain subfreez-

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ing conditions during irradiation, many processors attempt to process product quickly, or wrapped in insulating blankets, in order to minimize temperature change. The practice of placing dry ice (−76 °C) in conveyor totes along with product is also common. The purpose of this study was to determine the effect of product temperature, +5 °C to −20 °C, on the radiation resistance (D_{10}) of virulence plasmid containing *Y. enterocolitica* suspended in raw ground pork. The radiation resistance of virulence plasmid containing *Y. enterocolitica* suspended in ground pork, which was frozen in dry ice (−76 °C), was determined to provide processors with information relevant to actual commercial practices.

2. Materials and methods

Raw pork roast was purchased from a local market and ground to 3.2 mm pieces. The ground pork (20% fat) was then aliquoted (100 g) into No. 400 Stomacher bags (Tekmar, Inc., Cincinnati, OH) and vacuum-packaged to 0.26 mm Hg using a Multi-Vac A300 Vacuum-Packager (Kansas City, MO, USA). It was then overpacked in Mil-B Foil bags (Bell Fibre Corp., Columbus, GA, USA). In order to eliminate contaminating microorganisms the meat was radiation sterilized using the protocol of Thayer and Boyd (1995). The meat was then stored at −70 °C until ready for use.

Initial freezing point of the ground pork was determined by Differential Scanning Calorimetry (DSC). Pork samples for thermal analysis were thawed to 0°C. Approximately 50 mg were weighed into aluminum pans and placed in a Pyris 1 differential scanning calorimeter (Perkin-Elmer Corp., Norwalk, CT, USA) equipped with liquid nitrogen cooling and helium purge gas. After 2 min equilibration at −8 °C, the sample and an empty reference pan were heated at 0.3 °C/min. The initial freezing point was determined by calculating the onset temperature of the ice melting transition using the instrument's software.

Four *Y. enterocolitica* strains (S. Weager, FDA, Seattle, WA, USA) that contain the 70 kb large virulence plasmid, GER (serotype O:3), ATCC 51871 (serotype O:8), PT18-1 (serotype O:5,27) and EWM5 (serotype O:13) were utilized. Strains were propagated on Brain Heart Infusion Agar (BHA; Difco Laboratories, Detroit, MI, USA) at 27 °C and maintained at 0–2 °C until ready for use. Species verification was performed using Gram Negative Identification (GNI) of the Vitek Automicrobic System (bioMerieux, Inc., Hazelwood, MO, USA). Presence of the virulence plasmid-borne *VirF* gene was verified by Polymerase Chain Reaction (PCR; Fig. 2) as described by Bhaduri and Cottrell (1997). Presence of the large virulence plasmid was also verified by plasmid DNA isolation and visualization via agarose gel electrophoresis (data not shown).

The *Y. enterocolitica* strains were cultured independently in 100 ml Brain Heart Infusion Medium (BHI; Difco Laboratories) using baffled 500 ml Erlenmeyer flasks at 27 °C (150 rpm) for 18 h. The bacteria were then sedimented by centrifugation (3000 rpm for 30 min) and resuspended as a cocktail in a 10-fold reduced volume of Butterfield's Phosphate Buffer (BPB; Applied Research Institute, Newtown, CT, USA). The four strain cocktail was then diluted 1/10 into 100 g sterile ground pork and mixed for 90 s in a Stomacher Mixer (Tekmar, Inc.). The inoculated pork was then aliquoted (5 g) into No. 400 Stomacher bags, vacuum-packed to 26 mm Hg and stored at 0–2 °C until equilibrated to the irradiation temperature (15–30 min).

Vacuum-packaged samples, including unirradiated controls, were placed in a second No. 400 Stomacher bag and then placed in a preset isotemp bath for approximately 30 min for temperature (±0.5 °C) equilibration. The samples were then transferred to the temperature tempered irradiator chamber for irradiation. For irradiation at −76 °C the samples were tempered in dry ice for approximately 30 min prior to irradiation. The samples remained on dry ice during irradiation.

A Lockheed Georgia Company (Marietta, GA, USA) self-contained ^{137}Cs irradiator was used for all exposures. The radiation source consisted of 23 individually sealed source pencils in an annular array. The 22.9×63.5 cm cylindrical sample chamber was located central to the array when placed in the operating position. Inoculated samples were placed vertically and centrally in the sample chamber, using a 4 mm thick polypropylene bucket, to insure dose uniformity. The dose rate was 0.10 kGy/min.

The temperature during irradiation was maintained at the target by introduction of the gas phase from a liquid nitrogen source directly into the top of the sample chamber. Temperature was monitored by the use of two thermocouples, one placed centrally in the chamber and the other taped to the side of the sample bag. The absorbed dose was verified using temperature tempered 5 mm alanine pellets that were measured using a Bruker EMS 104 EPR Analyzer (Billerica, MA, USA). The ionizing radiation doses were 0.2, 0.4, 0.6, 0.8, and 1.0 kGy.

Following irradiation the samples were then assayed for colony-forming units (CFU) by standard pour plate method using BHA and 1/10 serial dilutions in BPB. Plates (three per dilution) were incubated at 37 °C for 1 day prior to scoring. CFU per plate, 30–300 per plate, were scored with a new Brunswick Scientific Biotran II colony counter. Unirradiated controls were routinely tested for the virulence plasmid associated trait of crystal violet binding (Bhaduri, Conway, & Lachica, 1987).

The means of triplicate plate counts of the treated samples (N) were divided by the average control plate

counts (No) to give a survivor ratio (N/No). The $\log_{10}$ (N/No) of the ratios were then used for determination of D_{10} values and other statistical analyses. D_{10} values were determined by the reciprocal of the slopes following linear regression as determined by least squares analysis (Sigma Plot, Version 5.0, Chicago, IL, USA). The predictive equation for determination of $\log_{10}$ survivor ratios was performed using Sigma Plot Version 5.0. Analysis of covariance (ANCOVA) was performed using Statistical Analysis Software (SAS, 1987 Version 6.12) (SAS Institute, Cary, NC, USA).

3. Results and discussion

A number of methods have been described to determine the initial freezing point of lean raw meats, which varied between −0.6 and −1.5 °C (Chang & Tao, 1981; Miles, Mayer, Morley, & Houska, 1997; Succar & Hayakawa, 1990). Mascheroni and Calvera (1980) estimated the initial freezing point of lean meat to be −1.1 °C, and the hard freeze point (>80% water frozen) to be −7 °C. The initial freezing point of the ground pork used in this study, −2.22 (±0.42)°C was determined empirically by DSC (Fig. 1). The predicted ice content, using the formula of Miles (1974), increased with decreasing temperature as shown in Fig. 1. Predicted ice contents were 55.6, 77.8, 85.2, 88.9 and 97.1% at −5, −10, −15, −20, and −76 °C, respectively.

PCR amplification of the virulence plasmid associated 591 bp *VirF* gene product from the four *Y. enterocolitica* strains used in the study (Lanes 1, 3, 5, 7), and from plasmid-less control strains (Lanes 2, 4, 6, 8), is shown in Fig. 2. The radiation resistance of the virulence plasmid containing *Y. enterocolitica* suspended in raw ground pork increased as temperature decreased. Survival curves are shown in Fig. 3. D_{10} values were 0.19, 0.19, 0.21, 0.40, 0.40, 0.38, and 0.55 kGy at 5, 0, −5, −10, −15, −20, and 76 °C, respectively. Statistical analysis using ANCOVA ($n=3$, $\alpha=0.01$) indicated that D_{10} values obtained at 5, 0, −5 °C were equivalent but significantly less than values obtained at −10, −15, −20, and −76 °C. D_{10} values obtained at −10, −15, −20, and −76 °C were equivalent as determined by ANCOVA ($n=3$, $\alpha=0.01$). Detailed results of the pair-wise comparisons are shown in Table 1.

Using $\log_{10}$ reduction data a predictive equation was developed (Fig. 4). The equation, which included data from +5°C to −20 °C, had a parabolic fit ($R^2=0.85$) where $\log_{10}$ reduction was equal to $-0.666-(2.955\times\text{Dose})-(0.069\times\text{Temp.})-(0.484\times\text{Dose}^2)-(0.001\times\text{Temp.}^2)$. When data from −76 °C was included ($R^2=0.85$) the equation was $\log_{10}$ reduction $=0.522-(3.197\times\text{Dose})-(0.056\times\text{Temp.})-(0.520\times\text{Dose}^2)-(0.001\times\text{Temp.}^2)$, the small change in formula reflecting the increase in D_{10} value observed at −76 °C.

The data obtained in this study is consistent with those obtained for other food-borne pathogens. That is, the radiation resistance of the microorganism is inversely related to product temperature. The D_{10} for *Escherichia coli* O157:H7 suspended in beef was 0.62 kGy at −15 °C versus 0.41 kGy at +5 °C (Lopez-Gonzalez et al., 1999). Thayer and Boyd (1995) found a D_{10} value for *L. monocytogenes* of 0.45 kGy in refrigerated (+5 °C) ground beef versus a D_{10} value of 1.21 kGy in beef frozen to a temperature of −20 °C. Thayer and Boyd (1992) found that *Staphylococcus aureus* was significantly more resistant to ionizing radiation at −20 °C than at +5 °C when suspended in mechanically deboned chicken meat. The radiation resistance, D_{10}, of *E. coli* O157:H7 was 0.39, 0.98 and 1.11 kGy in ground beef irradiated at +4, −20 and −76 °C, respectively (Thayer & Boyd, in press). The D_{10} of *S. aureus* was 0.48, 0.87 and 0.82 kGy in ground beef irradiated at

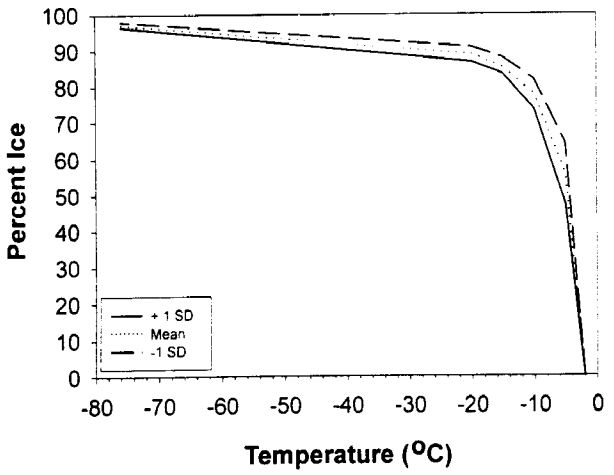

Fig. 1. Predicted ice content of raw ground pork (Mean±1 standard deviation) based on initial freezing point as determined by Differential Scanning Calorimetry ($n=3$).

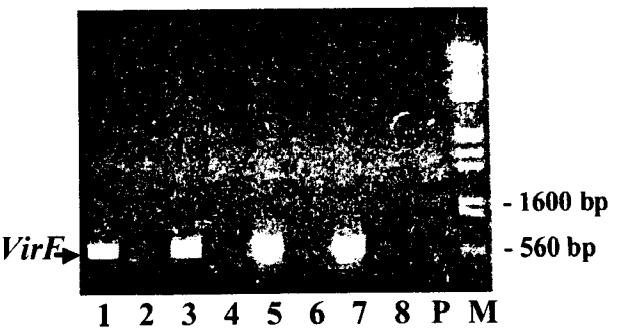

Fig. 2. Polymerase chain reaction amplification of 591 bp of the virulence plasmid-borne *VirF* gene from plasmid containing (P+) and plasmid-less (P−) *Yersinia enterocolitica* strains. Lane 1: GER P+, Lane 2: GER P−, Lane 3: ATCC 51871 (P+), Lane 4: ATCC 51871 (P−), Lane 5: PT18-1 (P+), Lane 6: PT18-1 (P−), Lane 7: EWM5 (P+), Lane 8: EWM5 (P−), Lane P: Primer (No Template) Control, Lane M: Lambda HindIII EcoRI molecular weight markers.

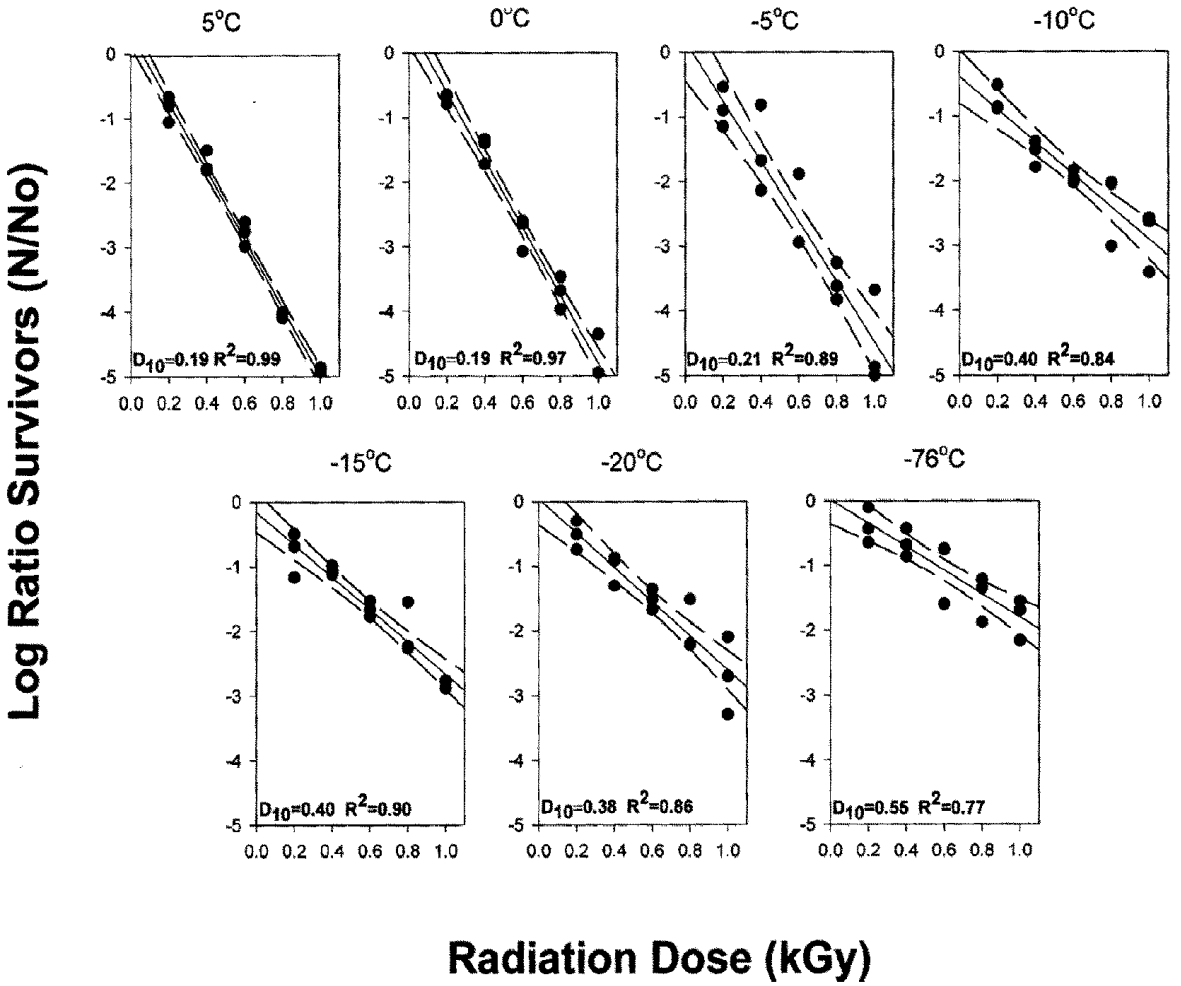

Fig. 3. Radiation resistance of *Yersinia enterocolitica* suspended in raw ground pork and maintained at various temperatures during irradiation. Linear regressions are shown as solid lines while 95% confidence intervals are shown as dashed lines. Each experiment was conducted independently three times.

Table 1
Pair-wise comparison of *Yersinia enterocolitica* D_{10} values as affected by product temperature

Temp. (°C)	5	0	−5	−10	−15	−20	−48	−76
5	X							
0	$P=0.78$	X						
−5	$P=0.26$	$P=0.26$	X					
−10	$P<0.01$*	$P<0.01$*	$P<0.01$*	X				
−15	$P<0.01$*	$P<0.01$*	$P<0.01$*	$P=0.99$	X			
−20	$P=0.01$ [a]	$P<0.01$*	$P<0.01$*	$P=0.73$	$P=0.71$	X		
−48	$P<0.01$*	$P<0.01$*	$P<0.01$*	$P=0.23$	$P=0.17$	$P=0.6$	X	
−76	$P<0.01$[a]	$P<0.01$*	$P<0.01$*	$P=0.17$	$P=0.14$	$P=0.18$	$P=0.06$	X

* Significantly different as determined by analysis of covariance ($n=3$, $\alpha=0.01$)

+4, −20 and −76 °C, respectively (Thayer & Boyd, in press).

The increased radiation resistance of microorganisms at subfreezing temperatures has been attributed to the lower a_w of meat at subfreezing temperatures and to decreased hydroxyl radical mobility following the radiolyis of water when in the frozen state (Bruns & Maxcy, 1979; Taub, Halliday, & Sevilla, 1979). Small changes in temperature surrounding the initial freezing point in meats can induce large changes in ice content (Miles, 1974). At $\leqslant -40$ °C, Mellor (1983) predicted that little or no free water was present in raw meats. The increase in *Y. enterocolitica* D_{10} to 0.55 kGy at −76 °C correlates with the predicted lack of free water.

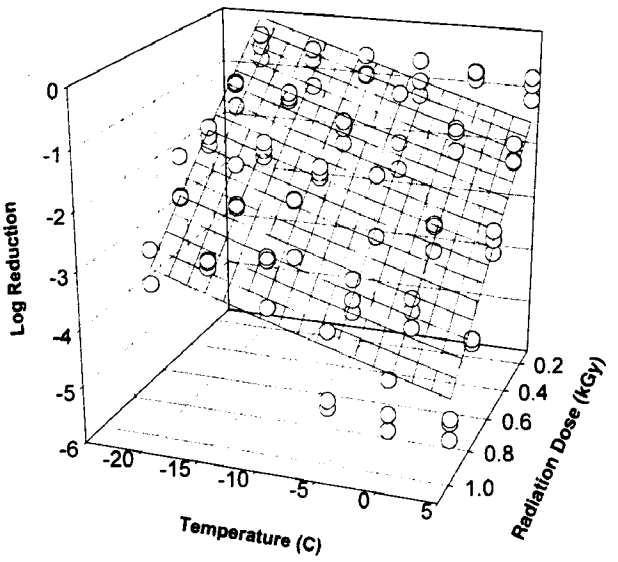

Fig. 4. A 3D mesh plot of $\log_{10}$ reduction data for *Yersinia enterocolitica* as a function of radiation dose and product temperature. The predictive equation followed a parabolic fit ($R^2 = 0.85$) where $\log_{10}$ reduction $= -0.666 - (2.955 \times \text{Dose}) - (0.069 \times \text{Temp.}) - (0.484 \times \text{Dose}^2) - (0.001 \times \text{Temp.}^2)$.

4. Conclusions

Detailed knowledge of product temperature profile is critical for effective ionizing radiation pasteurization of meat products. Pork products processed and packaged while refrigerated and then irradiated to reduce viable *Y. enterocolitica* five log $_{10}$ in number would require an ionizing radiation dose of 0.98 kGy. Hard frozen pork, $\leqslant -10$ °C, would require a dose of 2.0 kGy to produce a five log $_{10}$ reduction in *Y. enterocolitica*. Many commercial irradiation facilities lack temperature control capability. The commercial practice of using dry ice (−76 °C) in conveyor totes to keep product chilled or frozen during irradiation could produce similar results. An ionizing radiation dose of 2.75 kGy would be required to produce a five $\log_{10}$ reduction of *Y. enterocolitica* in a −76 °C product. Irradiation of partially or unevenly frozen pork could yield both microbiological and organoleptically undesirable results. It is the hope of these authors that the data presented here will assist processors in providing consumers microbiologically safe ionizing radiation treated pork products.

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